ORIGINAL ARTICLE

Trichosanthin-Stimulated Dendritic Cells Induce a Type 2 Helper T Lymphocyte Response Through the OX40 Ligand

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Abstract

Background: Trichosanthin (TCS) induces a type 2 helper T lymphocyte (T_H2) immune response that leads to the production of TCS-specific immunoglobulin (Ig) E and a subsequent allergic reaction in vivo. However, events immediately following treatment with TCS are poorly understood.
Objective: We aimed to investigate whether dendritic cells (DCs) are the initial mediators of T_H2 cell polarization induced by TCS and to investigate potential causative mechanisms.
Methods: DCs were cultured from purified human peripheral monocytes in the presence of granulocyte-macrophage colony-stimulating factor and interleukin (IL) 4. Flow cytometry was used to analyze cell surface antigen, intracellular cytokines, DC endocytic capacity, and apoptosis. The transcriptional profile for 120 genes in DCs was detected using oligonucleotide microarray analysis.
Results: TCS exerted a cytotoxic effect on DCs in a concentration-dependent manner. Although TCS alone did not induce full maturation of DC, it did so in the presence of tumor necrosis factor α and IL-1ß in vitro. TCS-stimulated DCs induced a decreased ratio of T_H1/T_H2 cells derived from naïve T cells and showed selective expression of OX40 ligand (OX40L) at both mRNA and protein levels. This induction was partially blocked by anti-OX40L antibody.
Conclusion: Our results imply that TCS-stimulated DCs polarize the development of T_H2 cells partially by means of the OX40L signal. Modulation of these DC will favor the alleviation of TCS-induced allergy.

Key words: Trichosanthin. Pertussis toxin. Dendritic cells. OX40 ligand.

Resumen

Antecedentes: El tricosantín (TCS) da lugar a una respuesta inmune Th2, que se expresa con la producción de inmunoglobulina E específica (IgE-TSC) y la posterior reacción alérgica *in vivo*. Sin embargo, los acontecimientos más tempranos de estos mecanismos son poco conocidos.
Objetivo: El objetivo fue investigar si son las células dendríticas (DC) las responsables iniciales de la polarización de las células Th2 inducida por el tratamiento con TCS y cuáles son los mecanismos responsables.
Métodos: Se cultivaron DC a partir de monocitos humanos periféricos purificados en presencia de GM-CSF e IL-4. Se utilizó citometría de flujo para analizar los antígenos de la superficie celular, las citocinas intracelulares, la capacidad de endocitosis de las DC y fenómenos de apoptosis. Se emplearon micromatrices de oligonucleótidos con un perfil transcripcional de 120 genes.
Resultados: El TCS ejerce un efecto citotóxico sobre las DC de una manera dosis-dependiente. El TCS, en solitario, no fue capaz de inducir la maduración completa de las DC mientras que sí lo hizo en presencia de TNF-α y IL-1ß. Las DC estimuladas con TCS originaron una disminución en la proporción de células Th1/Th2, derivadas de células T indiferenciadas, y expresaron selectivamente el ligando de OX40 (OX40L), tanto a niveles de ARNm como a niveles de proteína. Esta inducción fue bloqueada parcialmente por anticuerpos anti-OX40L.
Conclusiones: Nuestros resultados indican que las células dendríticas estimuladas con tricosantín polarizan el desarrollo de las células Th2 a través de la señal ejercida por el ligando de OX40. La modulación de estos fenómenos en las DC ayudará al control de la enfermedad alérgica inducida por tricosantín.

Palabras clave: Tricosantín. Toxina pertussis. Células dendríticas. Ligando de OX40.

Introduction

Tricosanthin (TCS) is a type I ribosome-inactivating protein isolated from the root of the Chinese medicinal herb *Trichosanthes kirilowii* (tian hua fen). The use of TCS to induce abortion dates back to 1000 years ago. Recent findings that TCS has antitumor and anti-HIV activity [1] have led to increased interest in the study of this protein. However, clinical use of TCS is limited owing to its potent allergenicity.

In mouse models, TCS has been shown to induce production of TCS-specific immunoglobulin E, the primary mediator in allergy [2]. TCS-induced immunization can upregulate the type 2 helper T cell (T_H2) cytokines interleukin (IL) 4 and IL-13 and inhibit expression of the T_H1 cytokine interferon (IFN) γ in murine splenocytes and mesenteric lymph node cells, suggesting that the T_H2 immune response is induced by TCS in vivo [3,12]. Moreover, the TCS-induced IgE response can be completely abolished by a blocking anti–IL-4 antibody [4]. Therefore, it can be postulated that TCS induces secretion of IL-4 and IL-13, which drive class switching to TCS-specific immunoglobulin (Ig) E, resulting in an allergic reaction. However, the cells targeted by TCS that promote development of T_H2 cells and the molecular mechanisms of this process have not been identified.

Dendritic cells (DCs) are the most powerful antigen-presenting cells for priming naïve T cells in vivo and play an essential role in T-cell differentiation. Upon contact with pathogens or proinflammatory factors, immature DCs (iDC) located at the site of infection are activated after taking up antigens and migrate to the T-cell region of lymphoid organs. In peripheral lymphoid tissues, DCs mature and present processed peptides to antigen-specific T cells in the context of major histocompatibility molecules. Costimulatory molecules on DCs are required to ensure correct activation of T cells through interaction with their counterparts on T cells. The differentiation of T cells into T_H1 or T_H2 effector cells is believed to be strictly regulated by cytokines secreted by DCs and membrane-bound molecules expressed on DCs [5,12].

Based on the known roles of DCs in initiating and directing an immune response, we hypothesized that the interaction between TCS and DCs leads to changes in the DC phenotype and functions that are necessary for the development of T_H2 cells. In this study, we show for the first time that TCS-stimulated DCs preferentially induce a decrease in the T_H1/ T_H2 ratio when cocultured with naïve T_H cells in vitro. Further experiments demonstrate that the high levels of expression of OX40L on DCs can contribute to the T_H2 immune response induced by TCS.

Materials and Methods

DC Cultures

Human monocytes were isolated from normal peripheral blood mononuclear cells (PBMC) by magnetic sorting with anti-CD14 microbeads (Miltenyi Biotech). Flow cytometry revealed that the purified cells were >97% $CD14^+$. iDCs were prepared from these monocytes by culture for 6 days in RPMI 1640 medium containing 10% fetal bovine serum (FBS) in the presence of granulocyte-macrophage colony-stimulating factor (500 U/mL, Peprotech) and IL-4 (10 ng/mL, Invitrogen/Biosource). At day 6, iDCs were treated for an additional 48 hours with the following factors alone or in combination: 10 ng/mL tumor necrosis factor (TNF) α (Invitrogen/Biosource) IL-1ß (10 ng/mL, Invitrogen/Biosource), 1 μg/mL pertussis toxin (PTX; Sigma), and indicated concentrations of TCS (Shanghai Jinshan Pharmaceutical Limited Company).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) Assay

MTT analysis was performed to evaluate cell viability, as described previously [6]. Briefly, 100 μL of iDC cell suspension (10^4 cells) was seeded into the wells of 96-well tissue culture plates containing the indicated concentrations of TCS. Control cells were not treated with TCS. After culturing for 48 hours, MTT (5 mg/mL; Sigma) was added to each well. Four hours later, the medium was removed and the formazan crystals produced by viable cells were dissolved with dimethyl sulfoxide. Absorbance at 490 nm was then measured. The viable cell number was expressed as a percentage of the control cells, measured as $100*A_{490\ nm,\ TCS\text{-}treated}/A_{490\ nm,\ control}$.

Measurement of Apoptosis

After 48 hours' incubation with the indicated concentrations of TCS, iDCs were harvested and stained with an Annexin V/ propidium iodide kit (Bender Medsystems GmbH). In brief, an aliquot of iDCs (50 μL of samples containing 2×10^5 iDCs) was added to 145 μL of binding buffer prepared according to the manufacturer's instructions combined with 5 μL of Annexin V–fluorescein isothiocyanate (FITC) and incubated at room temperature for 10 minutes. Samples were then washed once with the binding buffer and resuspended in 200 μL of binding buffer containing 1 mg/mL of propidium iodide. Flow cytometry analysis was performed within 1 hour of staining.

Cell Surface Antigen Analysis

Cell surface molecule expression was evaluated by staining DCs with the following mouse antihuman monoclonal antibodies (mAbs): FITC-labeled anti-CD80 (2D10.4, IgG1; eBioscience), phycoerythrin-labeled anti-CD83 (HB15e, IgG1; eBioscience), phycoerythrin-labeled anti-CD86 (IT2.2, IgG2b; eBioscience), and anti-OX40L (9H10, IgG1; prepared in our own laboratory) followed by phycoerythrin-labeled goat $F(ab')_2$ antimouse IgG (H+L) (Beckman Coulter). Cells were washed, resuspended in phosphate-buffered saline (PBS), and then analyzed on an EPICS Altra flow cytometer (Beckman Coulter).

Endocytosis Capacity

DCs were harvested, washed twice, and resuspended at 10^6 cells/mL in PBS. Two hundred microliters of the cell suspension were incubated with FITC-dextran solution (1 mg/mL Molecular Probes) for 60 minutes at 37°C. Background staining at 4°C was used as a negative control. After washing the cells 3 times with cold PBS, uptake of FITC-dextran by DC was analyzed by flow cytometry.

Allosensitization of Naïve T Cells by DCs

Highly purified $CD4^+CD45RA^+CD45RO^-$ naïve T cells (>96% assessed by flow cytometry) were obtained from normal human PBMCs using a $CD4^+CD45RO^-$ column kit (R&D Systems). Allosensitization was performed in 96-well tissue-culture plates by adding mitomycin C–treated DCs to 1×10^5 allogeneic naïve $CD4^+$ T_H cells in 200 µL of RPMI 1640 supplemented with 10% FBS at a 1:5 stimulator (DC)/responder (T cells) ratio. Mouse antihuman OX40L antibody (10 µg/mL; 9H10, mouse IgG1, prepared in our own laboratory), which is able to block the interaction between OX40L and the OX40 receptor [7], and its isotype control, mouse IgG1 (10 µg/mL, eBioscience), were added to some cocultures. After 4 days of culture, recombinant human IL-2 (20 U/mL, Invitrogen/Biosource) was added, and the cultures were expanded for an additional 9 days. On day 14, the T_H cells were washed and counted, and viable cells were restimulated with phorbol myristate acetate (10 ng/mL; Sigma) and ionomycin (1 µg/mL, Sigma) for 5 hours. A total of 10 µg/mL of brefeldin A (Sigma) was added for the last 3 hours of incubation. Intracellular cytokines were detected by flow cytometry using FITC-labeled anti-IFN-γ monoclonal antibody (mAb) (4S.B3, mouse IgG1; eBioscience) and phycoerythrin-labeled anti-IL-4 mAb (MP4-25D2, rat IgG1; eBioscience). T_H cells were identified as $CD3^+CD8^-$ using TC-anti-CD3 mAb (S4.1, mouse IgG2a; Invitrogen/Caltag Laboratories) and ECD-anti-CD8 mAb (3B5, mouse IgG2a; Invitrogen/Caltag Laboratories) because the surface expression of CD4 on T cells is markedly downregulated upon stimulation with phorbol 12-myristate 13-acetate/ionomycin [8]. Some T_H cells were restimulated with plate-bound anti-CD3 mAb (UCHT1, 0.5 µg/mL; Beckman Coulter) and anti-CD28 mAb (2F5, 2 µg/mL; prepared in our own laboratory). Supernatants were harvested after 24 hours, and the concentrations of IFN-γ and IL-4 were assayed using enzyme-linked immunosorbent assay (ELISA) kits from Invitrogen/Biosource.

Oligonucleotide Microarray Analysis

The microarray (SuperArray Inc.) contained oligonucleotide probes for 120 genes encoding 37 costimulatory molecules, 26 chemokines, 13 chemokine receptors, 13 cytokines, 18 cytokine receptors, 10 toll-like receptors, and 3 growth arrest and DNA damage gene 45 (*GADD45*) family members (Table 1) and was used to analyze the transcript profiles of these genes in DCs. iDCs were cultured from purified $CD14^+$ monocytes and then stimulated with TNF-α and IL-1ß or with TCS or PTX for 2 days to generate mature DCs. Total RNA was extracted from these cells and used as a template to generate biotin-labeled cRNA probes according to the manufacturer's instructions. The cRNA probes were denatured and hybridized at 60°C with the oligonucleotide array membranes,

Table 1. Genes (Total of 120) Detected Using Oligonucleotide Microarray Analysis

Gene Symbol	Gene Name	GenBank Accession No.
Costimulatory molecules and receptors		
CD28	Tp44	NM_006139
CD80	CD28LG/CD28LG1	NM_005191
CD86	B7-2/B70	NM_006889
CTLA4	CD/CD152	NM_005214
ICOS	AILIM/CD278	NM_012092
ICOSLG	B7-H2/B7H2	NM_015259
PDCD1	PD1/SLEB2	NM_005018
CD274	B7-H/B7H1	NM_014143
PDCD1LG2	B7DC/Btdc	NM_025239
CD276	B7-H3/B7H3	NM_025240
VTCN1	B7-H4/B7H4	NM_024626
TNFSF14	HVEML/LIGHT	NM_003807
TNFRSF14	ATAR/HVEA	NM_003820
LTBR	CD18/D12S370	NM_002342
BTLA	BTLA1	NM_181780
CD40LG	CD154/CD40L	NM_000074
CD40	Bp50/CDW40	NM_001250
TNFRSF4	ACT35/CD134	NM_003327
TNFSF4	CD134L/GP34	NM_003326
TNFSF9	4-1BB-L	NM_003811
TNFRSF9	4-1BB/CD137	NM_001561
TNFRSF11A	EOF/FEO	NM_003839
TNFSF11	ODF/OPGL	NM_003701
TNFRSF7	CD27/S152	NM_001242
TNFSF7	CD27L/CD27LG	NM_001252
TNFRSF8	CD30/D1S166E	NM_001243
TNFSF8	CD153/CD30L	NM_001244
ICAM1	BB2/CD54	NM_000201
ITGAL	CD11A/LFA-1	NM_002209
ICAM2	CD102	NM_000873
ICAM3	CD50/CDW50	NM_002162
CD209	CDSIGN/CLEC4L	NM_021155
SLAMF1	CD150/CDw150	NM_003037
CD2	SRBC/T11	NM_001767
CD58	LFA-3/LFA3	NM_001779
FAS	ALPS1A/APO-1	NM_000043
FASLG	APT1LG1/CD178	NM_000639
Chemokines and chemokine receptors		TGFB1
CCL2	GDCF-2/GDCF-2 HC11	NM_002982
CCL3	G0S19-1/LD78ALPHA	NM_002983
CCL4	ACT2/G-26	NM_002984
CCL5	D17S136E/RANTES	NM_002985
CCL7	FIC/MARC	NM_006273
CCL8	HC14/MCP-2	NM_005623
CCL11	SCYA11	NM_002986
CCL13	CKb10/MCP-4	NM_005408
CCL15	HCC-2/HMRP-2B	NM_032965
CCL16	CKb12/HCC-4	NM_004590
CCL17	ABCD-2/SCYA17	NM_002987
CCL18	AMAC-1/AMAC1	NM_002988
CCL19	CKb11/ELC	NM_006274
CCL20	CKb4/LARC	NM_004591
CCL21	6Ckine/CKb9	NM_002989
CCL22	ABCD-1/DC	NM_002990
CCL25	Ckb15/SCYA25	NM_005624
CCR1	CKR-1/CMKBR1	NM_001295
CCR2	CC-CKR-2/CCR2A	NM_000648
CCR3	CC-CKR-3/CKR3	NM_001837
CCR4	CC-CKR-4/CKR4	NM_005508
CCR5	CC-CKR-5/CCCKR5	NM_000579
CCR6	BN-1/CKR-L3	NM_004367
CCR7	BLR2/CDw197	NM_001838
CCRL1	CC-CKR-11/CCBP2	NM_016557

Table 1. Continued

Gene Symbol	Gene Name	GenBank Accession
*CXCL*1	GRO1/GROa	NM_001511
*IL*8	3-10C/AMCF-I	NM_000584
*CXCL*9	CMK/Humig	NM_002416
*CXCL*10	C7/IFI10	NM_001565
*CXCL*11	H174/I-TAC	NM_005409
*CXCL*12	PBSF/SCYB12	NM_000609
*CXCL*13	ANGIE/ANGIE2	NM_006419
*CXCL*16	CXCLG16/SR-PSOX	NM_022059
*CX3CL*1	ABCD-3/C3Xkine	NM_002996
IL8RA	C-C CKR-1/C-C-CKR-1	NM_000634
IL8RB	CDw128b/CMKAR2	NM_001557
*CXCR*3	CD183/CKR-L2	NM_001504
*CXCR*4	CD184/D2S201E	NM_003467
*BLR*1	CXCR5/MDR15	NM_001716
Cytokines and cytokine receptors		
IFNG	IFG/IFI	NM_000619
*IL*1*A*	IL-1A/IL1	NM_000575
*IL*1*B*	IL-1/IL1-BETA	NM_000576
IL2	IL-2/TCGF	NM_000586
IL4	BSF1/IL-4	NM_000589
*IL*6	BSF2/HGF	NM_000600
*IL*10	CSIF/IL-10	NM_000572
*IL*12A	CLMF/IL-12A	NM_000882
*IL*12B	CLMF/CLMF2	NM_002187
*IL*15	IL-15	NM_172175
*CED/DPD*1	NM_000660	
TNF	DIF/TNF-alpha	NM_000594
LTA	LT/TNFB	NM_000595
*IL*1*R*1	CD121A/D2S1473	NM_000877
*IL*1*R*2	IL1RB	NM_004633
IL2RA	CD25/IL2R	NM_000417
IL2RB	P70-75	NM_000878
IL2RG	CD132/IMD4	NM_000206
IL4R	CD124/IL4RA	NM_000418
IL6R	CD126/IL-6R-1	NM_000565
IL6ST	CD130/CDw130	NM_002184
*IL*10*RA*	CDW210A/HIL-10R	NM_001558
*IL*10*RB*	CDW210B/CRF2-4	NM_000628
*IL*12*RB*1	IL-12R-BETA1/IL12RB	NM_005535
*IL*12*RB*2	RP11-102M16.1	NM_001559
*IL*15*RA*	MGC104179	NM_002189
*IFNGR*1	CD119/IFNGR	NM_000416
*TGFBR*1	ACVRLK4/ALK-5	NM_004612
*TGFBR*2	HNPCC6/MFS2	NM_003242
*TNFRSF*1*A*	CD120a/FPF	NM_001065
*TNFRSF*1*B*	CD120b/TBPII	NM_001066
Toll-like receptors (TLRs)		
*TLR*1	DKFZp547I0610	NM_003263
*TLR*2	TIL4	NM_003264
*TLR*3	TLR3	NM_003265
*TLR*4	TOLL/hToll	NM_003266
*TLR*5	TIL3	NM_003268
*TLR*6	TLR6	NM_006068
*TLR*7	TLR7	NM_016562
*TLR*8	MGC119599	NM_016610
*TLR*9	TLR9	NM_017442
*TLR*10	MGC104967	NM_030956
Growth arrest and DNA damage gene 45 (GADD45)		
*GADD*45*A*	DDIT1/GADD45	NM_001924
*GADD*45*B*	MYD118	NM_015675
*GADD*45*G*	CR6/DDIT2	NM_006705

which were then washed and exposed to X-ray films using chemiluminescent substrates. The X-ray films were scanned, and the images acquired were inverted as grayscale TIFF files. The spots in the TIFF files were then digitized and analyzed with GEArray Analysis Suite software (SuperArray Inc). All raw signal intensities were corrected by subtraction of the background intensity of the spot containing plasmid DNA of pUC18 and then normalized by dividing them by the corrected signal intensity of the housekeeping gene (*ACTB*) on the same array. We considered a gene to be expressed when its normalized intensity value was ≥0.05.

Statistical Analysis

The statistical analysis was performed using a 2-tailed unpaired *t* test, and a *P* value <.05 was considered significant.

Results

Effect of TCS on Cell Viability of DCs

Since TCS has been reported to possess a cytotoxic effect on certain types of cells [9], we first investigated the viability of monocyte-derived iDCs after incubation for 48 hours with increasing concentrations of TCS.

The number of viable iDCs, as evaluated by the MTT assay, decreased significantly when the dose of TCS was above 400 ng/mL (Figure 1). Dual-color analysis of Annexin V and propidium iodide using flow cytometry revealed that TCS could induce early apoptosis of iDCs in a concentration-dependent manner. In addition, the number of late apoptotic iDCs increased markedly when TCS was increased to 5000 ng/mL, thus suggesting severe cell death in DCs (Figure 2).

Based on the above data, we decided to investigate the effects of 100 ng/mL of TCS on cells in all subsequent experiments, because TCS did not induce significant cytotoxicity in DC at this concentration.

Effect of TCS on DC Maturation

Full DC maturation is necessary for the activation and polarization of naïve T cells towards effector T_H cells, eg, T_H1 or T_H2 cells, which mediate cellular or humoral immunity, respectively. Therefore, we evaluated whether TCS could induce DC maturation. Our results revealed that TCS alone could not induce full maturation of DCs, since the maturation marker CD83 and the costimulatory molecules CD80 and CD86 were expressed at much lower levels on TCS-stimulated DCs than on DCs matured with the combination of TNF-α and IL-1ß (also known as maturation-inducing factors [MF]) (Figure 3A). Moreover, TCS did not significantly downregulate endocytosis of treated iDCs compared to nontreated iDCs, as demonstrated by the uptake of FITC-labeled dextran (Figure 3B). This observation further supported the fact that TCS-stimulated DCs were not fully

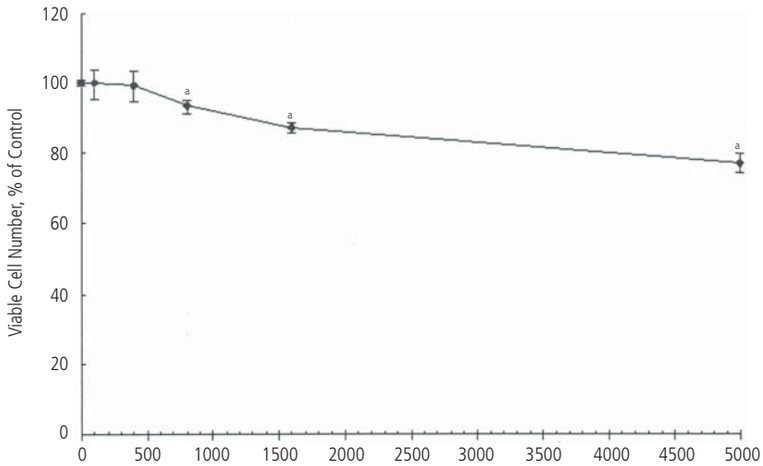

Figure 1. Cytotoxic effect of TCS on iDCs. iDCs were treated with the indicated concentrations of TCS (0, 100, 400, 800, 1600, and 5000 ng/mL) for 48 hours. The viable cell count was determined by MTT assay. Results are expressed as percentages of control values (without TCS treatment) and mean (SEM) (n=6).
[a]Significantly different values ($P<.001$) between iDCs treated with the indicated concentrations of TCS and controls.
TCS indicates trichosanthin; iDC, immature dendritic cell.

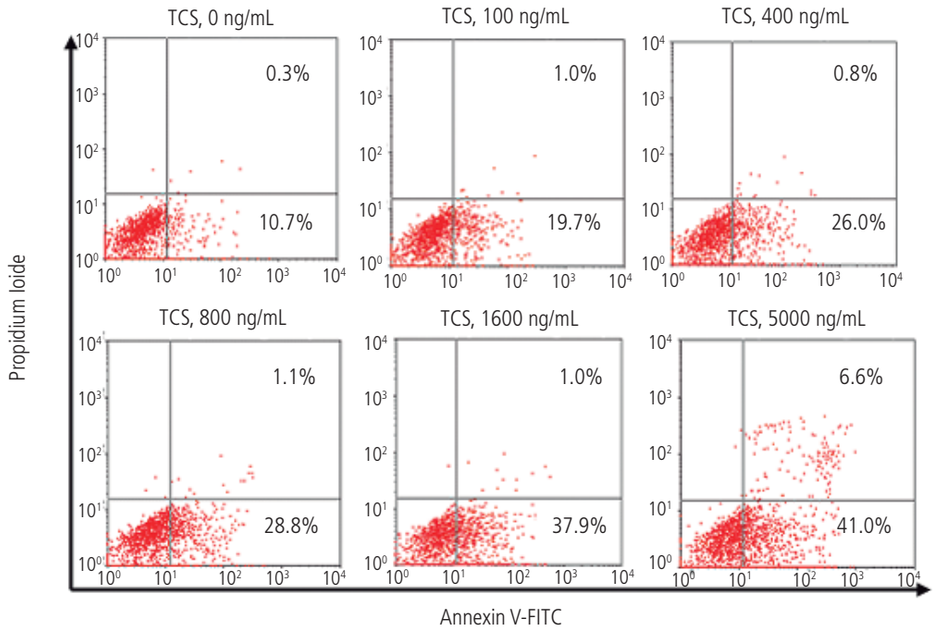

Figure 2. Flow-cytometric analysis of DC apoptosis. iDCs were incubated for 48 hours with the indicated concentrations of TCS (0, 100, 400, 800, 1600, and 5000 ng/mL, respectively) and cell apoptosis was measured using Annexin V-FITC/PI bivariate analysis. Numbers in the lower-right and upper-right quadrants refer to the percentages of early apoptotic DCs (annexin V+/PI–) and late apoptotic DCs (annexin V+/PI+), respectively. Results from 1 representative experiment of 3. DC indicates dendritic cell; iDC, immature DC; TCS, trichosanthin; PI, propidium iodide; FITC, fluorescein isothiocyanate.

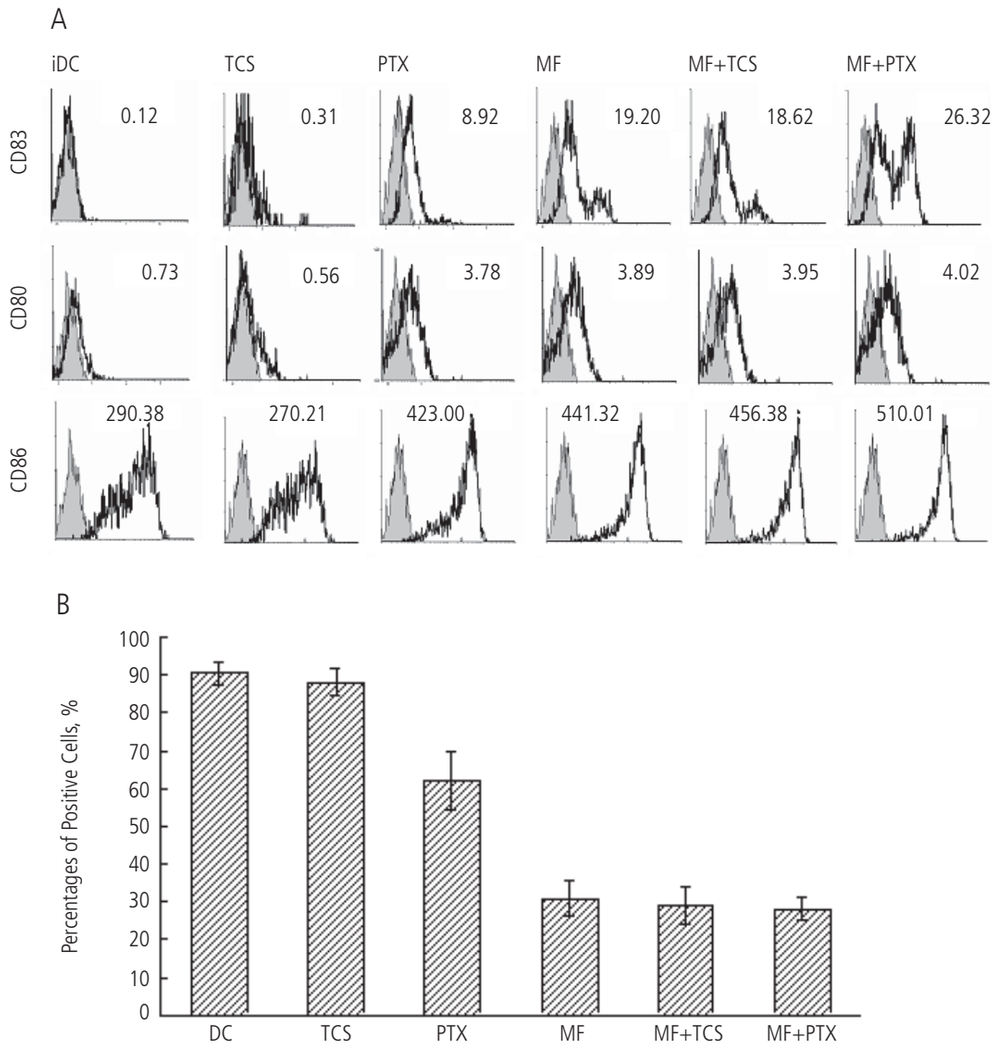

Figure 3. Effect of TCS on DC maturation. iDCs were incubated with TCS, PTX, MF, the combination of MF and TCS, and MF and PTX for 48 hours. A, Cells were analyzed for surface antigens CD83, CD80, and CD86 using flow cytometry and are shown as white histograms. The gray histograms represent the staining of the corresponding isotype controls. The results are representative of 4 experiments. The number in each graph represents the mean fluorescence intensity of positive cells. B, The uptake of FITC-labeled dextran by DCs was evaluated using flow cytometry. Results are expressed as mean (SEM) (n=4). TCS indicates trichosanthin; DC, dendritic cell; iDC, immature dendritic cell; PTX, pertussis toxin; MF, maturation factor; FITC, fluorescein isothiocyanate.

mature. Consistent with the findings of other studies [10], MF induced final maturation of DCs (Figure 3A and 3B).

PTX was used in this study, as it polarizes DCs to induce T_H1 effector cells [11] and can be a useful control for studying the effect of TCS on DC polarization. As shown in Figure 3, PTX alone induced maturation of DCs, which was evidenced to a certain extent by the induction of CD83 expression, upregulation of CD80 and CD86 (Figure 3A), and the downregulated uptake of FITC-labeled dextran (Figure 3B) compared to iDC. However, PTX-stimulated DCs were not as fully matured as MF-stimulated DCs (Figure 3A and 3B).

In order to study the effect of DCs on the development of T_H cells, the DCs must be fully activated. Since MF can induce final maturation of DCs and do not have a biased effect on the polarization of T_H cells [11], they were ideal factors to use in combination with TCS or PTX to generate fully activated DCs. In this study, we showed that MFs combined with TCS (MF+TCS) or PTX (MF+PTX) were able to induce full DC maturation (Figure 3).

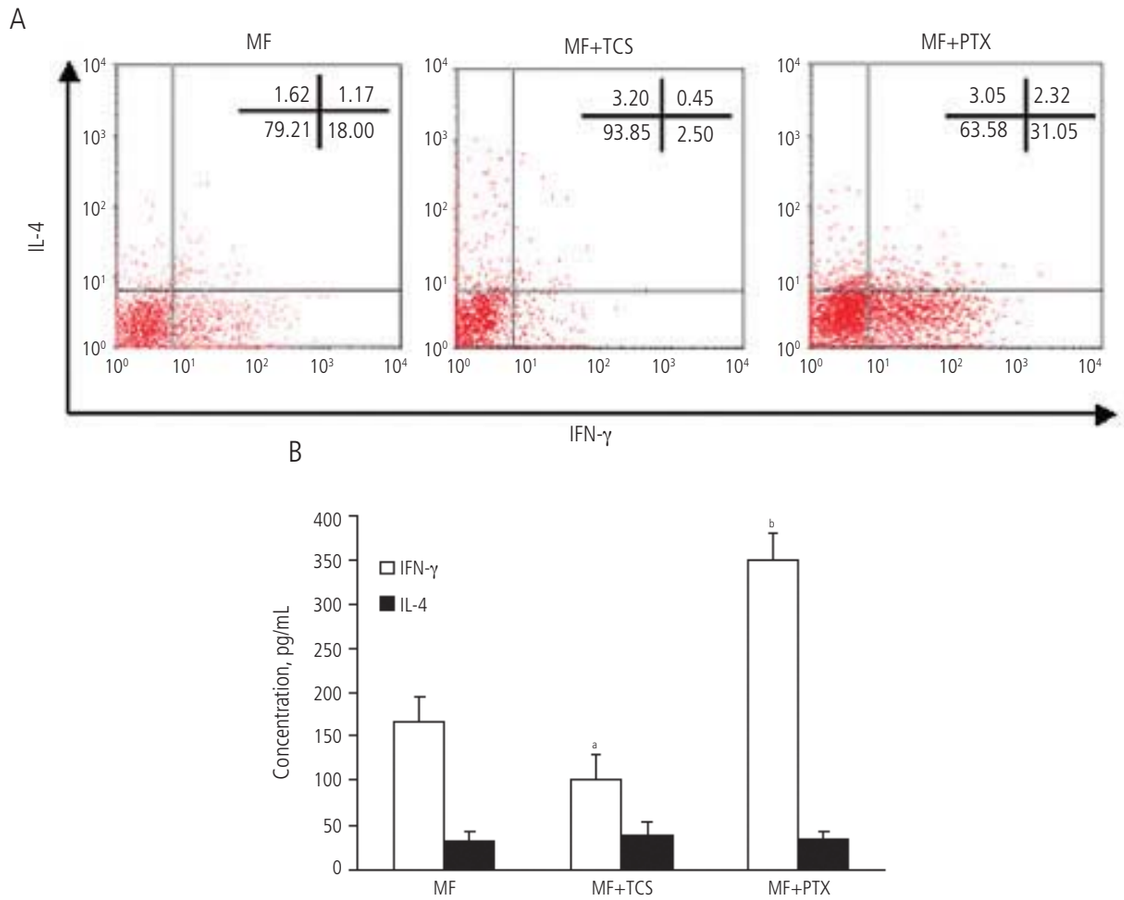

Figure 4. Effect of TCS on the ability of DCs to stimulate allogenous T_H lymphocytes. Human naïve $CD4^+$ T cells were cocultured for 4 days with allogeneic DCs and for a further 9 days for expansion of IL-2. A, Effector T_H cells were harvested and restimulated with phorbol 12-myristate 13-acetate and ionomycin for 5 hours. 10^5 cells were analyzed by flow cytometry for intracellular IFN-γ and IL-4 expression. The percentages of cell groups are indicated in the upper-right quadrant of each histogram. Data are from 1 experiment representative of 3 independent experiments. B, Effector T_H cells were harvested and restimulated with plate-bound anti-CD3 and anti-CD28 for 24 hours. IFN-γ (white bars) and IL-4 (black bars) were measured in culture supernatants using enzyme-linked immunosorbent assay. Data are the mean (SEM) values of 4 experiments. TCS indicates trichosanthin; DC, dendritic cell; IFN, interferon; IL, interleukin; PTX, pertussis toxin; MF, maturation factor.
[a]Significantly different values ($P<.05$) between DCs treated with MF+TCS and MF.
[b]Significantly different values ($P<.001$) between DCs treated with MF+PTX and MF.

Effect of TCS on the Capacity of DCs to Activate T_H Lymphocytes

DCs matured by MF, MF+TCS, and MF+PTX were used to stimulate allogenenic naïve T_H lymphocytes. The T_H1/T_H2 cytokine profiles of the resulting T_H cells were analyzed for intracellular IFN-γ and IL-4 using flow cytometry (Figure 4A). In comparison with MF and MF+PTX, the factors for polarizing DC into type 0 DCs (DC0) and type 1 DCs (DC1) [10], DCs matured with MF+TCS induced much lower percentages of IFN-γ-producing cells and similarly low levels of IL-4–producing cells (Figure 4A). Therefore, the T_H1/T_H2 ratio induced by DCs matured with MF+TCS was significantly lower than that induced by DCs matured with MF or MF+PTX, suggesting that MF+TCS–matured DCs preferentially induced T_H2 responses. This T_H2 response was confirmed by ELISA, since cocultures of allogeneic naïve T_H cells with DC matured with MF+TCS generated T_H cells with lower amounts of IFN-γ ($P<.05$) after restimulation with the combination of mAbs directed against CD3 and CD28 (Figure 4B) than T_H cells induced by DC matured with MF. The production of IL-4 by effector T_H cells induced by DCs matured with MF+TCS or MF was low-level and did not differ between these 2 treatments (Figure 4B). Thus, it can be postulated that DCs matured with MF+TCS became type 2 DCs (DC2), which favor the development of T_H2 cells. In contrast, DCs matured with MF+PTX resulted in DC1, which

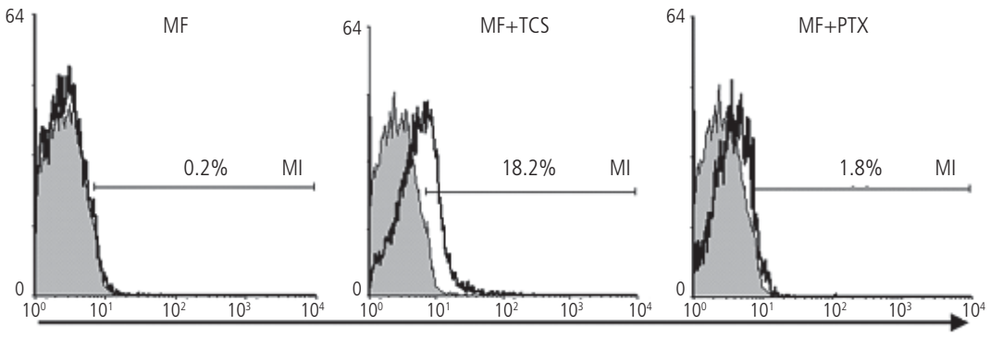

Figure 5. Expression of OX40L on DCs. Mature DCs were generated by treating iDCs with MF, MF+TCS, or MF+PTX for 48 hours and stained with anti-OX40L antibody followed by phycoerythrin-conjugated secondary antibody before being analyzed by flow cytometry. The blank curve in the histograms shows the specific expression of OX40L, whereas the solid curve shows the isotype control. Numbers above the gate (M1) refer to the percentage of OX40L-positive cells. Results form 1 representative experiment of 4.
DC indicates dendritic cell; iDC, immature dendritic cell; MF, maturation factor; TCS, trichosanthin; PTX, pertussis toxin.

favor the development of T_H1 cells, as shown by the increased number of IFN-γ–producing T_H cells and the higher level of IFN-γ secreted by effector T_H cells compared to DC matured with MF (Figure 4A and 4B).

Transcriptional Analysis of DC Subsets Using Oligonucleotide Microarray

In order to determine the molecules that could be involved in T_H cell polarization induced by various DCs, we used oligonucleotide microarrays to evaluate the gene expression profiles of the 3 DC subsets (DC0, DC1, and DC2). A gene was defined as differentially expressed when its transcripts were detectable (ie, both intensity values from 2 donors were more than 0.05) in 1 or 2 of the 3 DC subsets, or its transcripts were detectable in all 3 DC subsets but their levels showed >2-fold changes in the mean intensity for 2 donors between any 2 of the 3 DC subsets. Using the above criteria, 6 of the 120 genes were differentially expressed among the 3 DC subsets (Table 2). The mRNAs coding for IFNG (IFN-γ), CXCL9, and CXCL10 were expressed exclusively in DCs stimulated with MF+PTX. The transcript for *CCL19* was preferentially expressed by DC matured with MF+PTX, although it was present in all 3 DC subsets. Only 2 genes, *TNFSF4*, also known as *OX40L*, and *CCL20* were specifically transcribed in DCs exposed to MF+TCS. Our microarray results imply that distinct sets of genes are expressed in PTX- and TCS-stimulated DCs and that these genes may be essential for the development of T_H1 and T_H2 cells from naïve T_H cells when in contact with DCs matured with MF+PTX and MF+TCS, respectively.

OX40L Expressed by DCs Are Potentially Involved in T_H Cell Polarization

In order to confirm whether OX40L protein is also specifically expressed by DCs matured with MF+TCS, we detected OX40L expression using flow cytometry. Our results showed that DCs matured with MF+TCS expressed higher levels of OX40L than DCs stimulated with MF or MF+PTX (Figure 5). Thus, we proved that OX40L is characteristically expressed by MF+TCS–matured DCs at both mRNA and protein levels (Figure 5 and Table 2). Moreover, blocking of OX40L expressed on DCs matured with MF+TCS with anti-OX40L mAb resulted in a strong increase in the development of IFN-γ-producing T_H cells from naïve T_H cells, whereas when naïve T_H cells were stimulated with DC matured with MF or MF+PTX, no significant difference was observed in the development of IFN-γ–producing T_H cells in the presence of blocking anti-OX40L antibody or its isotype control, mouse IgG1 (Figure 6A). In addition, blocking antibody to OX40L

Table 2. Genes Differentially Expressed by Dendritic Cells Matured With MF, MF+TCS, or MF+PTX Detected Using Oligonucleotide Microarray[a]

Gene Symbol	MF	MF+TCS	MF+PTX
Costimulatory molecules			
*TNFSF*4	–	+	–
*Cytokines and cytokine receptor*s			
IFNG	–	–	++
Chemokines and chemokine receptors			
*CCL*19	+	+	++++
*CCL*20	–	++	–
*CXCL*9	–	–	++++
*CXCL*10	–	–	+

[a]Data represent the mean intensity (MI) from 2 donors. MI is categorized into the following groups: MI<0.05, –; 0.05≤MI<0.25, +; 0.25≤MI<0.50, ++; 0.50≤MI<0.75, +++; 0.75≤MI<1.00, ++++.

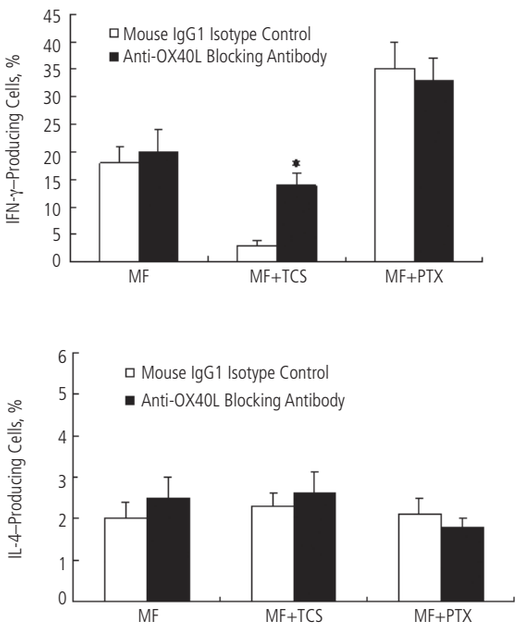

Figure 6. The role of OX40L expressed by DCs in T-cell polarization. Mature DCs were generated by treating iDCs with MF, MF+TCS, or MF+PTX for 48 hours and then cocultured with allogenous $CD4^+CD45RA^+$ naïve T_H lymphocytes in the presence of control antibody (white bars) or blocking anti-OX40L antibody (black bars). Flow cytometry was performed to evaluate the percentages of IFN-γ–producing cells (A) and IL-4–producing cells (B) among effector T_H cells. Results are expressed as mean (SEM) (n=4). DC indicates dendritic cell; iDC, immature dendritic cell; MF, maturation factor; TCS, trichosanthin; PTX, pertussis toxin; IFN, interferon; IL, interleukin.
[a]Significantly different values ($P<.01$) between cocultures in the presence of control antibody and in the presence of blocking anti-OX40L antibody.

did not affect the development of IL-4–producing T_H cells from naïve precursors induced by DCs matured with MF, MF+TCS, or MF+PTX (Figure 6B).

Discussion

Despite growing evidence that TCS induces an IgE response and subsequent allergic reaction through induction of T_H2 cell polarization [2,3,12], little is known about the roles of antigen-presenting cells, particularly DCs, in the generation of effector T_H cells induced by TCS. In this study, we show for the first time that TCS combined with MF induces complete maturation of DCs and that the effector DCs exhibit the characteristics of DC2, since they markedly inhibit the development of T_H1 cells from naïve precursors, resulting in a lower T_H1/T_H2 ratio compared to DCs matured with MF alone. Therefore, generation of polarized DCs may be a crucial step in the development of T_H2 cells induced by TCS.

In order to investigate the molecules potentially involved in T_H cell polarization induced by different DC subsets, we used the oligonucleotide microarray technique to analyze the transcriptional differences of some T_H cell differentiation–related proteins among DC0, DC1, and DC2. Notably, we found that OX40L, a costimulatory molecule, could be involved in the T_H2 cell polarization induced by DC2, because both OX40L mRNA and protein were selectively expressed in DC2 matured with MF+TCS. In addition, blocking of the OX40L–OX40 interaction between the DC2 and naïve T_H cells using an anti-OX40L antibody partially rescued the ability of effector T_H cells to produce IFN-γ. Our results are in line with previous findings that DCs stimulated with schistosomal egg antigen or thymic stromal lymphopoietin upregulated the expression of OX40L, which was instrumental in the induction of the subsequent T_H2 cell response [10,].

Surprisingly, our microarray analysis showed that the CC chemokine CCL20 was exclusively transcribed in DC2. This may not indicate a close relationship between CCL20 and T_H2 responses, as the expression of CCL20 is upregulated at the levels of mRNA and protein in monocyte-derived DCs matured with CD40L, a factor of DC1 polarization [14,15]. Thus, the role of CCL20 expressed by DCs in the development of effector T_H cells requires further investigation.

Our observations are consistent with the hypothesis that after induction by antigens, DCs are potentially involved in the polarization of T_H cells from their naïve precursors and both cytokines secreted by DCs and the membrane-bound molecules on DCs are candidates for transferring polarizing signals from DCs to antigen-specific T cells. Therefore, deviation of DC polarization in the opposite direction will be of significant help in alleviating the immunopathological effects of polarized T_H cells. In the case of TCS, the allergenicity effect of TCS may be overcome by applying blocking anti-OX40L antibody to inhibit the induction of DC2 and the development of subsequent T_H2 cell responses. Thus, the side effects of TCS will be significantly reduced, and TCS can then be widely used in clinical practice.

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